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(54) PROCESS FOR PREPARING FAT OR OIL CONTAINING UNSATURATED FATTY ACID

VERFAHREN ZUR HERSTELLUNG VON UNGESÄTTIGTE FETTSÄUREN ENTHALTENDEM FETT ODER ÖL

PROCEDE DE FABRICATION DE GRAISSE OU D'HUILE CONTENANT UN ACIDE GRAS INSATURE

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- **CHEMICAL ABSTRACTS, vol. 111, no. 7, 14 August 1989 (1989-08-14) Columbus, Ohio, US; abstract no. 55750, SHINMEN, YOSHIFUMI ET AL: "Production of arachidonic acid by Mortierella fungi. Selection of a potent producer and optimization of culture conditions for large-scale production" XP002124927 & APPL. MICROBIOL. BIOTECHNOL. (1989), 31(1), 11-16 ,1989,**
- **APPL. MICROBIOL. BIOTECHNOL., 1989, Vol. 31, No. 1, SHINMEN YOSHIFUMI et al., "Production of Arachidonic Acid by Mortierella Fungi", pages 11-16.**

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Description

Field of the Invention

[0001] The present invention relates to a process for producing unsaturated fatty acid-containing oils with a low 24,25-methylenecholest-5-en-3β-ol content using microorganisms belonging to the genus *Mortierella*, subgenus *Mortierella*.

Related Art

[0002] Microorganisms belonging to the genus *Mortierella*, subgenus *Mortierella* are known as microorganisms which produce unsaturated fatty acids such as arachidonic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid, and processes have been developed for efficient production of arachidonic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid by fermentation using these microorganisms (Japanese Unexamined Patent Publications No. 63-44891, No. 63-12290, No. 63-14696, No. 5-91887, No. 63-14697). In addition there is also known a process for producing Mead acid using mutant strains having reduced or defective in Δ12 desaturating activity, which are obtained by mutating microorganisms belonging to the genus *Mortierella*, subgenus *Mortierella* (Japanese Unexamined Patent Publication No. 5-91888).

[0003] Unsaturated fatty acids such as dihomo-γ-linolenic acid, arachidonic acid, eicosapentaenoic acid and Mead acid are precursors of prostaglandins, thromboxanes, prostacyclins, and leucotrienes which have powerful and versatile physiological activity, and much attention is therefore being directed to foods and animal feeds to which these are added.

[0004] For example, arachidonic acid is said to be a precursor of prostaglandins, thromboxanes, prostacyclins and leucotrienes which exhibit physiological activity including uterine muscle contracting and relaxing effects, vasodilator and antihypertensive effects, etc., and recently research has been rapidly progressing on docosahexaenoic acid (hereunder also abbreviated to "DHA") as an essential component particularly for infant development.

[0005] Specifically, Lanting et al. (LANCET, Vol.344, 1319-1322(1994)) have examined infants raised on breast milk and infants raised on infant powdered milk for 3 weeks or more after birth, with follow-up to 9 years of age, studying the incidence of minor damage to cranial nerves from a behavioral perspective, and have reported that the incidence of brain damage in children raised on infant powdered milk is twice that of children raised on breast milk. This shocking result suggests that higher unsaturated fatty acids such as DHA and arachidonic acid which are present in breast milk but virtually absent in infant powdered milk play a role in the development of the brain. Subsequent reports have also shown results suggesting that higher unsaturated fatty acids are connected with the development of the brain and retina.

[0006] Nevertheless, while unsaturated fatty acid-containing oils are considered to be highly safe, the issue of their microbial sources has prevented them from wide use throughout the world; meanwhile, in LIPIDS, Vol.27, No.6, 481-483 (1992), *Mortierella alpina* 1S-4 was reported to produce 24,25-methylenecholest-5-en-3β-ol which to that time was not known to occur naturally. Thus, it has been desired to develop unsaturated fatty acid-containing oils obtained from microorganisms belonging to the genus *Mortierella* subgenus *Mortierella* which can be more safely utilized for foods and animal feeds.

[0007] Shinmen et al in Applied Microbiology and Biotechnology, 1989, volume 31, No.1, pages 11-16 describes the assay of various *Mortierella* fungi for their productivity of arachidonic acid. The document includes reference to the culturing of various strains of *Mortierella* including strains from the subgenus *Mortierella*. The growth media which were used were described in earlier papers, one of them being a growth medium YM disclosed by Yamada et al Agric. Biol. Chem.51:785-790 (1987) comprising glucose polypeptone, yeast extract and malt extract, the other being a medium GY disclosed by Shimizu et al J.Am. Oil Chem. Soc. 65: 1455-1459 (1987) comprising glucose and yeast extract. Neither of those two earlier documents referred to culturing in a fermentor with aeration.

[0008] WO 92/13086 discloses processes for the production of arachidonic acid containing oils. The preferred micro-organism for cultivation is stated to be pythium insidiosum but Example 2 in the document mentions a culture of *Mortierella alpina* in a shake flask containing potato dextrose medium.

Disclosure of the Invention

[0009] It is therefore an object of the present invention to provide a microorganic oil which can be safely used in foods and animal feeds and which can economically and stably provide unsaturated fatty acids.

[0010] In order to solve the problem described above, the present inventors have searched for a process for efficient production of unsaturated fatty acid oils with a low content of 24,25-methylenecholest-5-en-3β-ol the use of which as a food is still unknown, and have studied in detail the relationship between the various medium components and sterol compositions; as a result they have found that it is possible to obtain oils with a low compositional ratio of 24,25-meth-

ylenecholest-5-en-3β-ol by using a nitrogen source derived from soybean for culturing of microoranisms belonging to the genus *Mortierella* subgenus *Mortierella*.

[0011] The present invention provides a process of production of unsaturated fatty acid-containing oil in which the composition ratio of 24,25-methylenecholest-5-en-3β-ol is not more than 35%, comprising culturing a microorganism belonging to the genus *Mortierella* subgenus *Mortierella* in a medium containing a nitrogen source derived from defatted soybean or physically or chemically processed defatted soybean, in a fermentor with aeration, and collecting said unsaturated fatty acid-containing oil from the cultured product.

[0012] In another aspect the invention provides an unsaturated fatty acid-containing oil having a 24,25-methylenecholest-5-en-3β-ol composition ratio of not more than 35% and obtainable by a process as above, containing from 20 to 54% arachidonic acid. The invention also provides an arachidonic acid-containing oil characterised by a 24,25-methylenecholest-5-en-3β-ol composition ratio of 35% or lower and an arachidonic acid content of from 20 to 54%, and obtainable by culturing a microorganism belonging to the genus *Mortierella* subgenus *Mortierella* in a fermentor with aeration.

[0013] The invention also relates to nutritive dietary supplement, immature infant formula, infant formula, baby food, pregnancy food products and animal feed containing oil as above and to processes for making such food stuffs by incorporating an oil as above.

Embodiment for Carrying Out the Invention

[0014] According to the invention, unsaturated fatty acids are fatty acids with at least 16 carbon atoms and at least one double bond, among which higher unsaturated fatty acids are generally fatty acids with at least 18 carbon atoms and at least two double bonds, and as examples there may be mentioned γ-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, eicosapentaenoic acid and Mead acid.

[0015] As examples of microorganisms belonging to the genus *Mortierella* subgenus *Mortierella* according to the invention there may be mentioned *Mortierella elongata, Mortierella exigua, Mortierella hygrophila, Mortierella alpina*, etc., and specifically there may be mentioned *Mortierella elongata* IFO8570, *Mortierella exigua* IFO8571, *Mortierella hygrophila* IFO5941, *Mortierella alpina* IFO8568, ATCC16266, ATCC32221, ATCC42430, CBS219.35, CBS224.37, CBS250.53, CBS343.66, CBS527.72, CBS529.72, CBS608.70, CBS754.68 and other cell lines.

[0016] These strains are all obtainable without restrictions from the Institute of Fermentation, Osaka (IFO), American Type Culture Collection (ATCC) and Centraalbureau voor Schimmelcultures (CBS). *Mortierella elongata* SAM0219 (FERM P-8703) (FERM-BP 1239) which was isolated from soil by the research group for the present invention, may also be used. These type culture cell lines or naturally occurring isolated cell lines may be used directly, but by growth and/or isolation at least once it is possible to obtain a natural mutant with different properties than the original cell line.

[0017] The microorganisms used according to the invention include mutant strains or recombinant strains of microorganisms belonging to the genus *Mortierella* subgenus *Mortierella* (wild strains), i.e. those designed either to give a higher unsaturated fatty acid content in the oil, a higher total oil content, or both, compared to the amount produced by the original wild strain, when cultured using the same substrate.

[0018] Also included are microorganisms designed to produce the same amount of unsaturated fatty acid as the wild strain through the efficient use of a substrate with an excellent cost effect. As examples there may be mentioned *Mortierella alpina* SAM1861 (FERM BP-3590) as a mutant strain defective in Δ12 desaturating activity and *Mortierella alpina* SAM1860 (FERM BP-3589) as a mutant strain defective in Δ5 desaturating activity.

[0019] The above-mentioned microorganisms belonging to the genus *Mortierella* subgenus *Mortierella* in the form of spores, hypha or a preculture obtained by previous culturing are inoculated into a liquid medium or solid medium and cultured. The carbon source used may be glucose, fructose, xylose, saccharose, maltose, soluble starch, molasses, glycerol, mannitol, citric acid, corn starch or any other conventional one, but glucose, maltose, fructose, corn starch, glycerol and citric acid are particularly preferred.

[0020] According to the invention, by using a nutrient source obtained from soybean as the nitrogen source it is possible to lower the compositional ratio of 24,25-methylenecholest-5-en-3β-ol in the oil.

[0021] The soybean-derived nitrogen source used for the invention is one with a nitrogen content of at least 2 wt%, preferably at least 3 wt% and more preferably at least 5% with respect to the total components except for water. The soybean-derived nitrogen source may be one or a combination of different types of defatted soybean or soybean subjected to heat treatment; acid treatment; alkali treatment; enzyme treatment; chemical modification; denaturation and/or renaturation by chemical and/or physical processing including heat treatment, acid treatment, alkali treatment, enzyme treatment, chemical modification, etc.; removal of a portion of the components with water and/or organic solvents; removal of a portion of the components by filtration and/or centrifugation; freezing; crushing; drying; sifting; etc., or a product of processing in the same manner as non-defatted soybean; as common candidates there may be mentioned soybean, defatted soybean, soybean flakes, edible soybean protein, okara, soy milk and roasted soybean flour (kinako), among which are particularly preferred heat-denatured defatted soybean, and especially heat-denatured de-

fatted soybean from which the ethanol-soluble components have been further removed.

[0022] When necessary one or more different additional nitrogen sources may also be added so long as the sterol composition is not notably affected, and examples include organic nitrogen sources such as peptone, yeast extract, malt extract, meat extract, casaminic acid, corn steep liquor and urea, and inorganic nitrogen sources such as sodium nitrate, ammonium nitrate and ammonium sulfate.

[0023] Also, when necessary trace nutrient sources may be used, including inorganic salts such as potassium phosphate, potassium dihydrogen phosphate and other phosphate salts, ammonium sulfate, sodium sulfate, magnesium sulfate, iron sulfate, copper sulfate, magnesium chloride, calcium chloride, etc., and vitamins.

[0024] According to the invention, accumulation of the unsaturated fatty acid of interest may be accelerated by accomplishing the culturing with addition of a substrate for the unsaturated fatty acid in the medium. The unsaturated fatty acid substrate used may be, for example, a hydrocarbon such as hexadecane or octadecane; a fatty acid such as oleic acid or linolic acid or a salt, for example a sodium or potassium salt thereof, or a fatty acid ester such as an ethyl ester, glycerol fatty acid ester or sorbitan fatty acid ester; or an oil such as olive oil, soybean oil, rapeseed oil, cottonseed oil or coconut oil, and these may be used alone or in combinations. The total amount of the substrate added is 0.001 to 10 wt%, and preferably 0.5 to 10 wt%, with respect to the medium. Any of these substrates may also be used as the sole carbon source for culturing.

[0025] The above-mentioned carbon sources, nitrogen sources, inorganic salts, vitamins and/or additives may be added to the medium prior to the start of culturing or to the culture broth during the cultivation. The medium components can be added all at once, or continuously or periodically through a few additions. The medium components may each be added alone or as a mixture. There are no particular restrictions on the concentrations of the medium components so long as growth of the cells is not inhibited. In practical usage, the carbon source should be at a concentration of 0.1 to 30 wt%, preferably 1 to 15 wt%, and the nitrogen source should be at a concentration of 0.01 to 10 wt%, and preferably 0.1 to 5 wt%.

[0026] The culturing temperature is 5 to 40°C, and preferably 20 to 30°C, and the unsaturated fatty acid may also be produced by growth of the cells by culturing at 20 to 30°C followed by continued culturing at 5 to 20°C. This manner of temperature control may also be employed to increase the yield of higher unsaturated fatty acids content in the fatty acids which are produced. The pH of the medium is 4 to 10, and preferably 5 to 8, and culturing with aeration and agitation, shake culturing or static culturing may be employed. The culturing is normally carried out for 2 to 20 days, preferably 5 to 20 days, and more preferably 5 to 15 days.

[0027] A fermenter, especially culturing fermenter with aeration and agitation or air-lift culturing fermenter may be used for submerged culturing with aeration to enable production with yields suited for unsaturated fatty acid-containing oils as commercial products. In such cases, the unsaturated fatty acid-containing oil can be even more efficiently produced by maintenance during the culturing to a glucose concentration of at least 0.3 wt% and/or an average glucose concentration of at least 0.5 wt%, preferably a glucose concentration of at least 0.5 wt% and/or an average glucose concentration of at least 0.7 wt%, and more preferably a glucose concentration of 0.5-5 wt% and/or an average glucose concentration of 0.7-3 wt%, for at least 3 days after the start of culturing. For example, arachidonic acid can be produced at 100 mg or more, and preferably 120 mg or more

[0028] to one gram of dry cells. Thus, an oil which is rich in the desired unsaturated fatty acid and low in 24,25-methylenecholest-5-en-3β-ol, is accumulated in large quantities in the cells.

[0029] The desired oil can be obtained according to a conventional method from the culture broth taken during production of the oil by the cell culturing or after its sterilization, the culture broth obtained at the end of culturing or after its sterilization, or the cultured cells collected from either, alternatively in dry form.

[0030] The desired oil may be collected from the cultured cells by the following method, for example.

[0031] After culturing is complete, the cultured cells are obtained from the culture broth by a conventional solid/liquid separation means such as centrifugation and/or filtration. The cultured cells are preferably washed, disrupted and dried. The drying may be accomplished by freeze drying or air drying. The dry cells are preferably subjected to extraction with an organic solvent preferably under a nitrogen stream. The organic solvent used may be ether, hexane, methanol, ethanol, chloroform, dichloromethane, or petroleum ether, and satisfactory results can also be obtained by alternate extraction with methanol and petroleum ether or by extraction using a chloroform-methanol-water monolayer system.

[0032] By removing the organic solvent from the extract under reduced pressure, it is possible to obtain an unsaturated fatty acid-containing oil at a high concentration. The extraction may also be accomplished using wet cells, instead of by the method described above. In this case there is used a water-compatible solvent such as methanol or ethanol, or a water-compatible mixed solvent including one of these with water and/or another solvent. The other procedures are the same as described above.

[0033] The oil obtained in this manner contains the unsaturated fatty acids in a state of triglycerides and phosphatidylcholine, phosphatidylethanolamine or phosphatidylinositol, but most of it is in the form of triglycerides. In order to separate and purify the unsaturated fatty acid-containing triglycerides from the unsaturated fatty acid-containing oil collected from the cultured product, a conventional method may be used for hexane extraction followed by deacidifi-

cation, decoloration, deodorization and degumming treatment, or cooling separation.

[0034] According to the invention, the compositional ratio of 24,25-methylenecholest-5-en-3β-ol is determined by the following method based on sterol composition analysis.

[0035] The sterol composition analysis will be explained first. A 30 to 80 mg portion of the oil is weighed out into a test tube with stopper, 4 mL of methanol and 1 mL of a 33% aqueous potassium hydroxide solution are added, and the stopper is fitted. After reaction for one hour while gently stirring at 80°C, the mixture is allowed to be cooled and the oil-soluble components are extracted with hexane. The resulting hexane solution is washed with water until a phenolphthalein indicator does not color the aqueous layer, and is then concentrated under a reduced pressure to obtain an analytical sample. The analytical sample is dissolved in a small amount of hexane and subjected to gas chromatography under the conditions listed in the table given below. By comparing the gas chromatogram with a commercially available desmosterol standard, the desmosterol peaks are identified.

[0036] The components which are detected within 0.8 to 2.0 times the retention time of desmosterol are the sterol components, and the peak areas of the gas chromatograms for all of the sterol components within the retention time are determined by a conventional method. The ratio of the peak area of each component to the sum of the total peak areas of the components is taken as the compositional ratio of each component. For example, the ratio of the peak area detected for desmosterol with respect to the sum of the total sterol area is the compositional ratio of desmosterol. 24,25-methylenecholest-5-en-3β-ol is detected in a retention time of 1.07 to 1.12 times the retention time of desmosterol. The ratio of the peak area detected for 24,25-methylenecholest-5-en-3β-ol with respect to the sum of all the peak areas is the compositional ratio of 24,25-methylenecholest-5-en-3β-ol. Column used: ULBON HR-1 (inner diameter: 0.25 mm, length: 25 m) Column temperature: 280°C Inlet and detector temperature: 300°C Carrier gas and gauge pressure, helium: 1.2 kg/cm^2 Make-up gas and flow rate, nitrogen: 70 mL/minute Detector: FID Split ratio: 20

[0037] The unsaturated fatty acid-containing oil of the invention has a 24,25-methylenecholest-5-en-3β-ol compositional ratio of 35% or less, preferably 33% or less, and more preferably 30% or less, and/or the 24,25-methylenecholest-5-en-3β-ol proportion is 1.2 or lower, preferably 0.9 or lower and more preferably 0.6 or lower with respect to the desmosterol present in the oil. Desmosterol is a component included with 24,25-methylenecholest-5-en-3β-ol in oils obtained by culturing microorganisms belonging to the genus *Mortierella* subgenus *Mortierella*, and it is known to be present in breast milk.

[0038] As an example of an unsaturated fatty acid-containing oil according to the invention there may be mentioned an arachidonic acid-containing oil with 20 to 54 wt% and preferably 30 to 50 wt% arachidonic acid with respect to the total fatty acids in the oil, and a 24,25-methylenecholest-5-en-3β-ol compositional ratio of 35% or lower, preferably 33% or lower and more preferably 30% or lower and/or a 24,25-methylenecholest-5-en-3β-ol proportion of 1.2 or lower, preferably 0.9 or lower and more preferably 0.6 or lower with respect to the desmosterol present in the oil.

[0039] The oil properties of the arachidonic acid-containing oil are such that the triglyceride content is 90% or greater, the moisture content is 0.1% or lower, the acid value is 0.5 or lower and the peroxide value is 5 or lower, while the color is ≤50 yellow and ≤10 by the Lovibond method in a 133.4 mm cell, and the fatty acid composition is 20 to 54%, with preferably 30 to 50% arachidonic acid, 0.2 to 0.7% myristic acid, 10 to 16% palmitic acid, 4 to 10% stearic acid, 5 to 15% oleic acid, 5 to 15% linolic acid, 1 to 5% γ-linolenic acid, 0.1 to 2% α-linolenic acid, 1 to 6% dihomo-γ-linolenic acid, 0 to 1% eicosapentoenoic acid and 2 to 7% lignoceric acid.

[0040] The oil is rich in the triglyceride form of arachidonic acid, and either contains no eicosapentoenoic acid or contains it only in a very trace amount, and is therefore desirable as a material for foods, and especially immature infant formula, infant formula, baby food and pregnancy food. The unsaturated fatty acid-containing oil of the invention can also be safely used in foods and animal feeds because of its low content of 24,25-methylenecholest-5-en-3β-ol, the edibility of which has not yet been established.

Examples

[0041] The present invention will now be explained in more detail by way of examples.

Example 1

[0042] Using *Mortierella elongata* IFO8570 as the arachidonic acid-producing cell line, 1400 L of a medium containing 2% glucose, 1% edible soybean protein (trade name: Esusan Meat, product of Ajinomoto Co.) and 0.1% rapeseed oil was placed in a 2000 L fermenter equipped with an agitator and aerator and culturing with aeration and agitation was initiated under conditions of 28°C temperature, 1.0 vvm aeration, 80 rpm agitation and 1.0 kg/cm^{2}G headspace pressure. The glucose concentration was kept at 1.5% by feeding glucose, and after culturing for 7 days the cells were recovered by filtration and subjected to oil extraction. As a comparative example, culturing and oil extraction were carried out in the same manner using 1% yeast extract instead of the edible soybean protein.

[0043] Upon analyzing the sterol composition of the resulting oil according to the procedure described above, des-

mosterol was detected at a retention time of about 9.6 minutes and 24,25-methylenecholest-5-en-3β-ol was detected at a retention time of about 10.5 minutes. In the comparative example, desmosterol was detected at a retention time of about 6.5 minutes and 24,25-methylenecholest-5-en-3β-ol was detected at a retention time of about 7.2 minutes. The results are shown in Table 1. Thus, an arachidonic acid-containing oil was obtained with a low compositional ratio of 24,25-methylenecholest-5-en-3β-ol.

Table 1

	24,25-methylene cholest-5-en-3β-ol compositional ratio (A)	Desmosterol compositional ratio (B)	A/B	Total sterol content*	Arachidonic acid content**
Example	30%	65%	0.46	1%	8%
Comp. Example	65%	27%	2.41	1%	9%

* Sterol content in oil

** Arachidonic acid content with respect to total fatty acids in oil

Example 2

[0044] *Mortierella alpina* CBS754.68 was used as the arachidonic acid-producing cell line, and 600 L of a medium containing 4% glucose, 1.3% roasted soybean flour (kinako), 0.2% yeast extract and 0.1% olive oil was placed in a 1000 L fermenter equipped with an agitator and aerator, for culturing with aeration and agitation for 5 days under conditions of 24°C temperature, 1.0 vvm aeration, 100 rpm agitation and 0.5 kg/cm^{2}G headspace pressure, followed by filtration and drying to recover the cells and hexane extraction to obtain an oil. As a comparative example, culturing was carried out in the same manner using a medium of 4% glucose, 1.5% yeast extract and 0.1% olive oil to obtain an oil. In both the example and the comparative example, 1% glucose was added on the 2nd day of culturing.

[0045] Upon analyzing the sterol composition of the resulting oil according to the procedure described above, desmosterol was detected at a retention time of about 10.2 minutes and 24,25-methylenecholest-5-en-3β-ol was detected at a retention time of about 11.2 minutes. In the comparative example, desmosterol was detected at a retention time of about 6.4 minutes and 24,25-methylenecholest-5-en-3β-ol was detected at a retention time of about 7.1 minutes. The results are shown in Table 2. Thus, an arachidonic acid-containing oil was obtained with a low compositional ratio of 24,25-methylenecholest-5-en-3β-ol.

Table 2

	24,25-methylene cholest-5-en-3β-ol compositional ratio (A)	Desmosterol compositional ratio (B)	A/B	Total sterol content*	Arachidonic acid content**
Example	25%	53%	0.47	1.2%	48%
Comp. Example	68%	16%	4.25	1.1%	46%

* Sterol content in oil

** Arachidonic acid content with respect to total fatty acids in oil

Example 3

[0046] *Mortierella alpina* ATCC32221 and *Mortierella alpina* ATCC42430 were used as arachidonic acid-producing cell lines, and each was cultured. After placing 25 L of a medium containing 4% glucose, 1.2% defatted soy powder, 0.2% potassium hydrogen phosphate and 0.1% soybean oil in a 50 L fermenter equipped with an agitator and aerator, culturing with aeration and agitation was carried out for 5 days under conditions of 28°C temperature, 1.0 vvm aeration, 300 rpm agitation and 1.0 kg/cm^{2}G headspace pressure, followed by filtration and drying to recover the cells and hexane extraction to obtain an oil from the recovered cells.

[0047] As a comparative example, culturing was carried out in the same manner using a medium of 4% glucose, 1.2% beer yeast powder, 0.2% potassium hydrogen phosphate and 0.1% rapeseed oil to obtain an oil. In both the example and the comparative example, 1% glucose was added on the 2nd day of culturing. The sterol composition of the resulting oil was analyzed according to the procedure described above. The results are shown in Table 3. Thus, an arachidonic acid-containing oil was obtained with a low compositional ratio of 24,25-methylenecholest-5-en-3β-ol.

Table 3

	24,25-methylene cholest-5-en-3β-ol compositional ratio (A)	Desmosterol compositional ratio (B)	A/B	Total sterol content*	Arachidonic acid content**
Mortierella alpina ATCC32221	5%	67%	0.07	0.9%	25%
Comp. Example	37%	28%	1.32	0.8%	20%
Mortierella alpina ATCC42430	5%	35%	0.14	0.9%	18%
Comp. Example	40%	25%	1.60	1.0%	18%

* Sterol content in oil

** Arachidonic acid content with respect to total fatty acids in oil

Example 4

[0048] Using *Mortierella alpina* CBS754.68 as the arachidonic acid-producing cell line, 1400 L of a medium containing 2% glucose, 1.5% soybean protein and 0.1% soybean oil was placed in a 2000 L fermenter equipped with an agitation and aeration, and culturing with aeration and agitation was initiated under conditions of 24°C temperature, 1 vvm aeration, 80 rpm agitation and 200 kPa headspace pressure. The glucose concentration was kept at 0.5 to 1.5% by feeding glucose, and after culturing for 7 days the cells were recovered by filtration. After drying the cells, they were extracted with hexane, the extracted oil was subjected to deacidification, decoloration and deodoration, and 0.05% tocopherol was added as an antioxidant. The resulting oil was analyzed and found to have the following composition.

Analysis results

[0049]

Triglyceride content: 95.6%
Moisture: 0.04%
Acid value: 0.08
Peroxide value: 2.16
Color (Lovibond method, 133.4 mm cell): yellow: 20.1, red: 1.4
Fatty acid composition:

arachidonic acid	44.4%
myristic acid	0.6%
palmitic acid	14.6%
stearic acid	8.8%
oleic acid	6.3%
linolic acid	10.2%
γ-linolenic acid	3.2%
α-linolenic acid	0.8%
dihomo-γ-linolenic acid	5.2%
eicosapentoenoic acid	0.2%
lignoceric acid	4.8%

Total sterol content : 1.0%
24,25-methylenecholest-5-en-3β-ol compositional ratio: 24%
Desmosterol compositional ratio: 67%

Example 5

[0050] The arachidonic acid-containing oil obtained in Example 4 was appropriately mixed with fish oil and vegetable oil to obtain an essential fatty acid-adjusted oil. In addition to the essential fatty acid-adjusted oil, the raw materials and components listed below were prepared for formulation of 100 kg of powdered infant formula. After dissolving, mixing and refining the raw materials according to conventional methods, they were sterilized, concentrated and homogenized, and then spray dried to obtain powdered infant formula.

Raw materials and components	
casein	5.6 kg
whey protein concentrate	24.0 kg
essential fatty acid-adjusted oil (composed mainly of linolic acid, α-linolenic acid)	25.0 kg
arachidonic acid content	80 g
docosahexaenoic acid content	25 g
eicosapentaenoic acid content	10 g
saccharides (lactose and oligosaccharides)	43.4 kg
minerals and vitamins	2 kg
TOTAL	100 kg

Claims

1. A process of production of unsaturated fatty acid-containing oil in which the composition ratio of 24,25-methylene-cholest-5-en-3β-ol is not more than 35%, comprising culturing a microorganism belonging to the genus *Mortierella* subgenus *Mortierella* in a medium containing a nitrogen source derived from defatted soybean or physically or chemically processed defatted soybean, in a fermentor with aeration, and collecting said unsaturated fatty acid-containing oil from the cultured product.

2. A process according to claim 1 in which said nitrogen source derived from defatted soybean provides a nitrogen content of at least 2 wt% with respect to the total components excluding water.

3. A process according to claim 2 in which said nitrogen source provides a said nitrogen content of at least 5 wt%.

4. A process according to claim 1, 2 or 3 in which the defatted soybean of the nitrogen source has been processed by heat treatment; acid treatment; alkali treatment; enzyme treatment; chemical modification; denaturation and/or renaturation by chemical and/or physical processing including a said treatment; removal of a portion of the components with water and/or organic solvents; removal of a portion of the components by filtration and/or centrifugation; freezing; crushing; drying; and/or sifting.

5. A process according to claim 1, 2 or 3 in which said nitrogen source derived from defatted soybean is from defatted soybean which has been subjected at least to heat denaturation.

6. A process according to any one of the preceding claims in which said cultured product is a culture broth taken during production of the oil by the cell culturing or after its sterilisation, a culture broth obtained at the end of culturing or after sterilisation thereof, or cultured cells collected from either of these, optionally in dry form.

7. A process according to any one of the preceding claims in which said fermentor is equipped with an agitator and air sparger or is an air-lift fermentor.

8. A process according to any one of the preceding claims in which the culturing is carried out while maintaining a glucose concentration of at least 0.3 wt% and/or an average glucose, concentration of at least 0.5 wt%, for at least 3 days after the start of culturing.

9. A process according to any one of the preceding. claims in which the culturing is carried out for a period of from 2 to 20 days.

10. A process according to any one of the preceding claims in which unsaturated fatty acid in said oil is γ-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, eicosapentaenoic acid and/or mead acid.

11. A process according to claim 10 in which the oil contains from 20 to 54% arachidonic acid and its compositional ratio of 24,25-methylenecholest-5-en-3β-ol is not more than 35%.

12. A process for making a nutritive dietary supplement, an immature infant formula, an infant formula, baby food, pregnancy food product or animal feed, **characterised by** incorporating an arachidonic acid containing oil produced in accordance with claim 11 into said supplement, formula, food or feed.

13. An unsaturated fatty acid-containing oil having a 24,25-methylenecholest-5-en-3β-ol composition ratio of not more than 35% and obtainable by a process according to any one of the preceding claims, containing from 20 to 54% arachidonic acid.

14. An arachidonic acid-containing oil **characterised by** a 24,25-methylenecholest-5-en-3β-ol composition ratio of 35% or lower and an arachidonic acid content of from 20 to 54%, and obtainable by culturing a microorganism belonging to the genus *Mortierella* subgenus *Mortierella* in a fermentor with aeration.

15. An arachidonic acid-containing oil according to claim 14 in which said culture used a soybean-derived nitrogen source.

16. An arachidonic acid-containing oil according to claim 13, 14 or 15, further **characterised in that** the 24,25-methylenecholest-5-en-3β-ol compositional ratio is in a proportion of not more than 1.2 with respect to the desmosterol compositional ratio.

17. An arachidonic acid-containing oil according to any one of claims 13 to 16 which is a microbial oil obtained from a said microorganism of the genus *Mortierella* subgenus *Mortierella*.

18. A nutritive dietary supplement comprising an arachidonic acid-containing oil according to any one of claims 13 to 17.

19. An immature infant formula, infant.formula, baby food or pregnancy food product comprising an arachidonic acid-containing oil according to any one of claims 13 to 17.

20. An animal feed comprising an arachidonic acid-containing oil according to any one of claims 13 to 17.

Patentansprüche

1. Verfahren zur Erzeugung eines ungesättigte Fettsäurenhaltigen Öls, worin das Zusammensetzungsverhältnis von 24,25-Methylencholest-5-en-3β-ol nicht mehr als 35 % beträgt, umfassend die Kultivierung eines Mikroorganismus, der zur Gattung Mortierella, Untergattung Mortierella gehört, in einem Medium enthaltend eine Stickstoffquelle, abgeleitet von entfetteter Sojabohne oder physikalisch oder chemisch verarbeiteter entfetteter Sojabohne in einem Fermenter mit Belüftung und Sammeln des ungesättigten Fettsäurenhaltigen Öls aus dem kultivierten Produkt.

2. Verfahren gemäß Anspruch 1, worin die Stickstoffquelle, abgeleitet von entfetteter Sojabohne, einen Stickstoffgehalt von mindestens 2 Gew.% im Hinblick auf die Gesamtbestandteile außer Wasser bereitstellt.

3. Verfahren gemäß Anspruch 2, worin die Stickstoffquelle einen Stickstoffgehalt von mindestens 5 Gew.% bereitstellt.

4. Verfahren gemäß Anspruch 1, 2 oder 3, worin die entfettete Sojabohne der Stickstoffquelle durch Wärmebehandlung; Säurebehandlung; Alkalibehandlung; enzymatische Behandlung; chemische Modifikation; Denaturierung und/oder Renaturierung durch chemische und/oder physikalische Verarbeitung einschließlich einer solchen Behandlung verarbeitet wurde; Entfernung eines Teils der Bestandteile mit Wasser und/oder organischen Lösungsmitteln; Entfernung eines Teils der Bestandteile durch Filtration und/oder Zentrifugation; Einfrieren; zerkleinern; Trocknen und/oder Sieben.

5. Verfahren gemäß Anspruch 1, 2 oder 3, worin die Stickstoffquelle, abgeleitet von entfetteter Sojabohne, aus entfetteter Sojabohne stammt, die mindestens einer Wärmedenaturierung unterzogen wurde.

6. Verfahren gemäß einem der vorstehenden Ansprüche, worin das kultivierte Produkt eine Kulturbrühe ist, die während der Erzeugung des Öls durch die Zellkultivierung genommen wurde oder nach der Sterilisierung, eine Kulturbrühe, erhalten nach Abschluß der Kultivierung oder nach deren Sterilisierung oder kultivierte Zellen, gesammelt von einer der obigen, optional in getrockneter Form.

7. Verfahren gemäß einem der vorstehenden Ansprüche, worin der Fermenter mit einer Rührvorrichtung ausgerüstet ist sowie mit einer Luft-Sprühvorrichtung oder ein Airlift-Fermenter ist.

8. Verfahren gemäß einem der vorstehenden Ansprüche, worin die Kultivierung durchgeführt wird, während eine Glucosekonzentration von mindestens 0,3 Gew.% und/oder eine durchschnittliche Glucosekonzentration von mindestens 0,5 Gew.% für mindestens 3 Tage nach Beginn der Kultivierung aufrechterhalten wird.

9. Verfahren gemäß einem der vorstehenden Ansprüche, worin die Kultivierung für eine Zeitspanne von 2 bis 20 Tagen durchgeführt wird.

10. Verfahren gemäß einem der vorstehenden Ansprüche, worin die ungesättigte Fettsäure in dem Öl γ-Linolensäure, Dihomo-γ-linolensäure, Arachidonsäure, Eikosanpentaensäure und/oder Metsäure (mead acid) ist.

11. Verfahren gemäß Anspruch 10, worin das Öl 20 bis 54 % Arachidonsäure enthält und das Zusammensetzungsverhältnis von 24,25-Methylencholest-5-en-3β-ol nicht mehr als 35 % beträgt.

12. Verfahren zur Herstellung eines diätetischen Nahrungsergänzungsmittels, einer Formel zur Ernährung von unreifen Kleinkindern, einer Kleinkind-Formel, Babynahrung, Nahrungsmittel für die Schwangerschaft oder Tierfutter, **gekennzeichnet durch** Inkorporation eines Arachidonsäure-haltigen Öls erzeugt gemäß Anspruch 11 in das Ergänzungsmittel, die Formel, das Nahrungsmittel oder das Futtermittel.

13. Ungesättigte Fettsäuren-haltiges Öl mit einem 24,25-Methylencholest-5-en-3β-ol Zusammensetzungsverhältnis von nicht mehr als 35 % und erhältlich durch ein Verfahren gemäß einem der vorstehenden Ansprüche, enthaltend 20 bis 54 % Arachidonsäure.

14. Arachidonsäure-haltiges Öl, **gekennzeichnet durch** ein 24,25-Methylencholest-5-en-3β-ol Zusammensetzungsverhältnis von 35 % oder weniger und einen Arachidonsäure-Gehalt von 20 bis 54 %, und erhältlich **durch** Kultivierung eines Mikroorganismus, der zur Gattung Mortierella, Untergattung Mortierella gehört in einem Fermenter mit Belüftung.

15. Arachidonsäure-haltiges Öl gemäß Anspruch 14, worin die Kultur eine von Sojabohnen abgeleitete Stickstoffquelle verwendet.

16. Arachidonsäure-haltiges Öl gemäß Anspruch 13, 14 oder 15, weiter **dadurch gekennzeichnet, daß** das 24,25-Methylencholest-5-en-3β-ol Zusammensetzungsverhältnis in einem Verhältnis von nicht mehr als 1,2 im Hinblick auf das Desmosterol Zusammensetzungsverhältnis steht.

17. Arachidonsäure-haltiges Öl gemäß einem der Ansprüche 13 bis 16, wobei es sich um ein mikrobielles Öl handelt, erhalten von dem Mikroorganismus der Gattung Mortierella, Untergattung Mortierella.

18. Diätetisches Nahrungsergänzungsmittel, umfassend ein Arachidonsäure-haltiges Öl gemäß einem der Ansprüche 13 bis 17.

19. Formel für unreife Kleinkinder, Kleinkinder-Formel, Babynahrung oder Nahrungsprodukt für die Schwangerschaft, umfassend ein Arachidonsäure-haltiges Öl gemäß einem der Ansprüche 13 bis 17.

20. Tierfutter, umfassend ein Arachidonsäure-haltiges Öl gemäß einem der Ansprüche 13 bis 17.

Revendications

1. Procédé de production d'huile contenant des acides gras insaturés dans lequel le poucentage en 24,25-méthylènecholest-5-en-3β-ol n'est pas supérieur à 35%, comprenant la mise en culture d'un microorganisme appartenant au genre *Mortierella* sous-genre *Mortierella* dans un milieu contenant une source d'azote dérivé de graine de soja dégraissée ou de graine de soja dégraissée par un traitement physique ou chimique, dans un fermenteur muni d'aération, et la collecte de ladite huile contenant des acides gras insaturés à partir du produit cultivé.

2. Procédé selon la revendication 1 dans lequel ladite source d'azote dérivé de graine de soja dégraissée fournit une teneur en azote d'au moins 2% en poids par rapport à la totalité des composants à l'exception de l'eau.

3. Procédé selon la revendication 2 dans lequel ladite source d'azote fournit une dite teneur en azote d'au moins 5% en poids.

4. Procédé selon la revendication 1, 2 or 3 dans lequel la graine de soja dégraissée de la source d'azote a été traité par un traitement thermique ; par un traitement par un acide; par un traitement par un alcali; par un traitement par un enzyme ; par une modification chimique ; par une dénaturation et/ou une renaturation par un traitement chimique et/ou physique y compris ledit traitement ; par le retrait d'une partie des composants avec de l'eau et/ou des solvants organiques; par le retrait d'une partie des composants par filtration et/ou centrifugation; par congélation ; par concassage ; par séchage ; et/ou par criblage.

5. Procédé selon la revendication 1, 2 ou 3 dans lequel ladite source d'azote dérivé de graine de soja dégraissée est dérivée de graine de soja dégraissée qui a été soumise au moins à une dénaturation par la chaleur.

6. Procédé selon l'une quelconque des revendications précédentes dans lequel ledit produit cultivé est un bouillon de culture pris pendant la production de l'huile par la mise en culture des cellules ou après sa stérilisation, un bouillon de culture obtenu à la fin de la culture ou après la stérilisation de celui-ci, ou des cellules cultivées recueillies de l'un ou l'autre de ceux-ci, facultativement sous une forme sèche.

7. Procédé selon l'une quelconque des revendications précédentes dans lequel ledit fermenteur est muni d'un agitateur et d'un pulvérisateur d'air ou est un fermenteur à agitation par circulation d'air.

8. Procédé selon l'une quelconque des revendications précédentes dans lequel la culture est mise en oeuvre tout en maintenant une concentration en glucose d'au moins 0,3% en poids et/ou une concentration moyenne en glucose d'au moins 0,5% en poids pendant au moins 3 jours après le début de la culture.

9. Procédé selon l'une quelconque des revendications précédentes dans lequel la culture est mise en oeuvre pendant une durée de 2 à 20 jours.

10. Procédé selon l'une quelconque des revendications précédentes dans lequel l'acide gras insaturé dans ladite huile est l'acide γ-linolénique, l'acide dihomo-γ-linolénique, l'acide arachidonique, l'acide eïcosapentaénoïque et/ou l'acide d'hydromel.

11. Procédé selon la revendication 10 dans lequel l'huile contient de 20 à 54% d'acide arachidonique et le pourcentage de composition en 24,25-méthylènecholest-5-en-3β-ol n'est pas supérieur à 35%.

12. Procédé pour la fabrication d'un supplément diététique, d'une formulation pour les nourrissons immatures, d'une formulation pour les nourrissons, d'un aliment pour bébés, d'un produit alimentaire pour les femmes enceintes ou d'un aliment pour les animaux, **caractérisé par** l'incorporation d'un acide arachidonique contenant de l'huile produite selon la revendication 11 dans ladite ou ledit supplément, formulation, aliment ou produit alimentaire.

13. Huile contenant des acides gras insaturés ayant une proportion de 24,25-méthylènecholest-5-en-3β-ol non supérieure à 35% et pouvant être obtenue par un procédé selon l'une quelconque des revendications précédentes, contenant de 20 à 54% d'acide arachidonique.

14. Huile contenant de l'acide arachidonique **caractérisée par** une proportion de 24,25-méthylènecholest-5-en-3β-ol de 35% ou inférieure à 35% et une teneur en acide arachidonique allant de 20 à 54%, et pouvant être obtenue par la mise en culture d'un microorganisme appartenant au genre *Mortierella* sous-genre *Mortierella* dans un

fermenteur muni d'aération.

15. Huile contenant de l'acide arachidonique selon la revendication 14 dans laquelle ladite culture a employé une source d'azote dérivé de graine de soja.

16. Huile contenant de l'acide arachidonique selon la revendication 13, 14 ou 15, **caractérisée en outre en ce que** le pourcentage de 24,25-méthylènecholest-5-en-3β-ol par rapport au pourcentage de desmostérol est en une proportion non supérieure à 1,2.

17. Huile contenant de l'acide arachidonique selon l'une quelconque des revendications 13 à 16 qui est une huile microbienne obtenue à partir d'un dit microorganisme du genre *Mortierella* sous-genre *Mortierella*.

18. Supplément diététique nutritionnel comprenant une huile contenant de l'acide arachidonique selon l'une quelconque des revendications 13 à 17.

19. Formulation pour les nourrissons immatures, formulation pour les nourrissons, aliment pour bébés ou produit alimentaire pour les femmes enceintes comprenant une huile contenant de l'acide arachidonique selon l'une quelconque des revendications 13 à 17.

20. Aliment pour animaux comprenant une huile contenant de l'acide arachidonique selon l'une quelconque des revendications 13 à 17.